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THE STEADY-STATE TRANSPORT OF CATIONIZED FERRITIN BY ENDOTHELIAL CELL VESICLES

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SUMMARY

1. The steady-state transfer of cationized ferritin by endothelial cell vesicles has been investigated quantitatively using electron microscopy. Single capillaries from the mesenteries of decerebrated frogs were perfused *in vivo* with solutions containing 3–5 g 100 ml^{-1} cationized ferritin or cationized ferritin (3–5 g 100 ml^{-1}) and bovine albumin (1 g 100 ml^{-1}). Perfusions lasted between 60 and 240 s, at which time the tissues were fixed *in situ* with osmium tetroxide.

2. Measurements of the free diffusion co-efficient of cationized ferritin in the presence and absence of 1 g 100 ml^{-1} albumin ($0{\cdot}400 \pm 0{\cdot}09 \times 10^{-6}$ cm^2 s^{-1} and $0{\cdot}361 \pm 0{\cdot}08 \times 10^{-6}$ cm^2 s^{-1}, respectively) were not significantly different which suggests that albumin does not bind to cationized ferritin. Together they yielded a value for the Stokes–Einstein radius of cationized ferritin of 5·59 nm, which was not significantly different from that of native ferritin.

3. Examination of transverse sections of perfused capillaries showed a layer of cationized ferritin molecules (> 26 nm thick) close to the luminal surface of the endothelial cell wall, in both the presence and absence of albumin. Estimates of the concentration of cationized ferritin within the layer showed it to be approximately twice that of the perfusate concentration, confirming that cationized ferritin binds and concentrates at the cell surface.

4. When no albumin was present in the cationized ferritin perfusate, all the luminal vesicles (those open to the luminal cell surface) were labelled with molecules of cationized ferritin. The mean number of ferritin molecules per labelled luminal vesicle (F/N_L) was $5{\cdot}0 \pm 0{\cdot}7$, a value close to that predicted from the concentration of cationized ferritin in the layer if it was assumed that the whole of the vesicle volume was available to molecules of cationized ferritin, i.e. that cationized ferritin could penetrate the cell coat lining the vesicles as it does that covering the luminal cell surface.

5. Few cytoplasmic vesicles ($0{\cdot}26 \pm 0{\cdot}04$) and abluminal vesicles ($0{\cdot}07 \pm 0{\cdot}03$) were labelled with cationized ferritin in the absence of albumin. The mean number of cationized ferritin molecules per labelled vesicle in both vesicle populations was also low. In addition, labelled cytoplasmic vesicles $F/N_L = 1{\cdot}87 \pm 0{\cdot}33$) always contained significantly fewer ferritin molecules than labelled luminal vesicles ($F/N_L = 5{\cdot}0 \pm 0{\cdot}7$). These findings offer further support for the fusion model of the steady-state transfer of ferritin by endothelial cell vesicles (Clough & Michel, 1981) and are not consistent with the translocation of labelled luminal vesicles across the

cell. They also suggest that cationized ferritin binds to the cell coat lining the vesicles, and is unavailable for transfer during transient fusions between vesicles.

6. The presence of albumin in the cationized ferritin perfusate reduced the fractional labelling of all three vesicle populations to one third of their values in its absence. It also reduced the mean number of ferritin molecules per labelled vesicle at all three sites in the cell. It is suggested that albumin reduces the volume of distribution of cationized ferritin within the vesicles either by competing with cationized ferritin for the same binding sites within the cell coat, or by simply occupying space within the extracellular matrix.

INTRODUCTION

The experiments described in this paper were designed to gain further information concerning the steady-state transfer of macromolecules by endothelial cell vesicles. In recent years the traditional view of vesicles as 'ferry boats' (Palade, 1960; Mayerson, Wolfram, Shirley & Wasserman, 1960, Renkin, 1964), moving with their contents between the cell surfaces, has been questioned (Bundgaard, Frøkjaer-Jensen & Crone, 1979; Frøkjaer-Jensen, 1980; Clough & Michel, 1981, 1982). Quantitative estimates of the steady-state labelling of vesicles with native ferritin in individually perfused frog mesenteric capillaries have suggested that native ferritin molecules are transported through the cell as the result of a series of fusions and separations of neighbouring vesicles, the fusions lasting long enough for their contents to mix (Clough & Michel, 1981). Entry of native ferritin into the vesicles open to the capillary lumen appears, however, to be restricted, possibly by the ruthenium red staining layer, or glycocalyx, which has been shown by Luft (1966) and others (Shirahama & Cohen, 1972) to cover the luminal cell surface and line the endothelial cell vesicles. The result of this exclusion of ferritin from the luminal vesicles in such experiments is that rather low levels of cytoplasmic and abluminal labelling are sometimes seen which are difficult to interpret (Clough & Michel, 1981).

In the present study an attempt has been made to overcome this. Clough & Michel's experiments have been repeated, using a polycationic form of ferritin which binds to the cell coat (Danon, Goldstein, Marikovsky & Skutelsky, 1972; Simionescu & Simionescu, 1978, 1981; Sprague, Frankel, Hagens, Wilhelm & Schwartz, 1981) and in some preparations, heavily labels the endothelial cell vesicles (Pelikan, Gimbrone & Cotran, 1979; Sprague *et al.* 1981). The binding of cationized ferritin to the endothelium of singly perfused frog mesenteric capillaries, *in vivo*, has been investigated using electron microscopy and the steady-state labelling of endothelial cell vesicles with molecules of cationized ferritin quantitatively assessed. The techniques used were those of Loudon, Michel & White (1979), modified by Clough & Michel (1981). The effect of albumin on the uptake of cationized ferritin by the vesicles was also investigated, Clough & Michel (1981) having shown that the uptake of native ferritin by luminal vesicles is greatly reduced when albumin is present in the ferritin perfusate.

Measurements of the free diffusion co-efficient of cationized ferritin have been made, in both the presence and absence of albumin, and the Stokes–Einstein radius of cationized ferritin estimated to ensure that neither cationization nor the presence of albumin alters the molecular radius of the tracer molecule. In this way a direct

comparison may be made between the patterns of labelling of vesicles with cationized ferritin seen in the present study and those seen by Clough & Michel (1981) using native ferritin. A preliminary report of part of this work has been made to the Physiological Society (Clough, 1981).

METHODS

Solutions. In all the experiments, capillaries were perfused with a solution of 3–5 g cationized ferritin in 100 mmol NaCl l^{-1}. The cationized ferritin was made from cadmium-free native horse spleen ferritin (Sigma Chemical Co., 10% (w/v) in 150 mmol NaCl l^{-1}) by the method described by Danon *et al.* (1972). After dialysation against frog Ringer for 24 h, the cationized ferritin was concentrated by ultrafiltration through an Amicon UM5-membrane to give a final concentration of between 3 and 5 g cationized ferritin 100 ml^{-1} Ringer (for Ringer composition see Clough & Michel, 1981). The concentration of the cationized ferritin was estimated colorimetrically using the commercially produced native ferritin as a standard. The isoelectric point (pI) for both native and cationized ferritin was estimated using isoelectric focusing on polyacrylamide gels and found to be 4·5 and > 10·5 respectively. In some experiments bovine serum albumin 1 g 100 ml^{-1} was added to the cationized ferritin solution.

All solutions were filtered through a Millipore filter (pore diameter 0·22 μm) prior to use, and their pH adjusted to between 7·2 and 7·4.

The buffers used in the preparation of the tissues for electron microscopy were diluted to 70% of the usual strength to approximate in tonicity with frog tissues.

Experimental procedures. All the capillaries used in this study were from the mesenteries of *Rana pipiens* whose brains had been destroyed by pithing. The mesenteries were prepared for transillumination as previously described (Levick & Michel, 1973) and the mesentery was continuously superfused with a frog Ringer solution, cooled to 15–17 °C. The procedure for cannulating and perfusing capillaries has been described elsewhere (Levick & Michel, 1973; Clough & Michel, 1981). The capillaries were perfused for periods of between 60 and 240 s with solutions containing cationized ferritin 3–5 g 100 ml^{-1} or cationized ferritin and albumin 1 g 100 ml^{-1} at a pressure of 30 cmH_2O. After this time, when a steady-state should be achieved in the capillary lumen (Loudon *et al.* 1979; Clough & Michel, 1981), the superfusion drip was switched off and the tissues were flooded with ice-cold 2% osmium tetroxide. The tissues were post-fixed *in situ* with 6% glutaraldehyde for a further 30 min and then dissected from the animal and left in glutaraldehyde in cacodylate buffer for a further 12–24 h. The tissues were prepared for electron microscopy as described by Loudon *et al.* (1979). Transverse sections of the perfused capillaries (60–80 nm thick) were mounted on uncoated copper grids and after grid staining with lead, examined using a Jeol JEM-100CX transmission electron microscope.

Assessment of labelling. Measurements of the thickness of the layer of cationized ferritin molecules adhering to the cell surface were made from photomicrographs of transeverse sections of the perfused capillaries (× 79,000). The number of cationized ferritin molecules within the layer, expressed as the number of molecules per μm^3, was estimated by counting ferritin molecules in the layer and assuming a section thickness of 80 nm. Endothelial cell vesicles were divided into three types, luminal, cytoplasmic and abluminal (see Loudon *et al.* 1979) and the labelling of the vesicles expressed as (i) the fraction of vesicles in a given population containing one or more ferritin molecules, N_L/N_T; and (ii) the mean number of ferritin molecules per labelled vesicle, F/N_L, where N_T is the total number of vesicles counted, N_L is the number labelled and F is the number of ferritin molecules within the vesicles counted. All these values were corrected for sectioning losses and the 95% confidence limits of the mean values of N_L/N_T and F/N_L estimated (see Clough & Michel, 1981, for details).

Measurement of diffusion co-efficient of cationized ferritin. Diffusion co-efficients, D, for cationized ferritin alone, and for cationized ferritin and bovine serum albumin 1 g 100 ml^{-1}, in frog Ringer, were measured using a simplified Fürth microdiffusion cell (Fürth, 1945; Levick & Michel, 1973; Clough & Michel, 1981). The hydrodynamic molecular radius of cationized ferritin, a_{es}, was estimated from the relationship:

$$a_{es} = \frac{RT}{6D\pi\eta N},$$

where η is the viscosity of water at 20 °C (room temperature), R and T are the gas constant and absolute temperature, and N is the number of molecules per mol (Avogadro Number).

The values for a_{es} for cationized ferritin in the presence and absence of albumin were compared with those previously obtained for native ferritin in the same diffusion cell (Clough & Michel, 1981). A possible interaction of ferritin molecules with molecules of albumin was also investigated, by the use of gel electrophoresis. The mobilities of both native and cationized ferritin in the presence and absence of bovine serum albumin 1 g 100 ml^{-1}, through polyacrylamide gels at pH 7·29, were compared by measuring the distances run by the ferritin molecules down the gels after application of a constant current for a known length of time.

RESULTS

Diffusion coefficient for cationized ferritin

Ten estimates of the diffusion coefficient of cationized ferritin in Ringer solution were made in five experiments in the absence of albumin, and nineteen estimates in six experiments where cationized ferritin diffused through Ringer containing albumin 1 g 100 ml^{-1}. The mean value for D in the presence of albumin was $0{\cdot}400 \pm 0{\cdot}09 \times 10^{-6}$ cm^2 s^{-1} ($n = 19$) and in the absence of albumin was $0{\cdot}361 \pm 0{\cdot}08 \times 10^{-6}$ cm^2 s^{-1} ($n = 10$) (mean ± S.E. of the mean). The Stokes–Einstein radii calculated from these values of D were 5·36 ± 0·17 nm and 5·94 ± 0·15 nm respectively, and 5·59 ± 0·30 nm when based on the mean of all twenty-nine estimates of D. The mean value of a_{es} for native ferritin, estimated from its diffusion coefficient in the same cell, was 6·12 ± 0·31 nm. These data reveal that cationization of ferritin does not alter its molecular radius. They further suggest that there is little interaction between cationized ferritin molecules and albumin molecules in Ringer solution. This finding is confirmed when the mobility of cationized ferritin is measured electrophoretically. The presence of albumin appears not to affect either the mobility of cationized ferritin, nor that of native ferritin, in a constant current field.

Labelling of endothelial cell coat

The general morphology of the endothelium of capillaries perfused with solutions containing cationized ferritin appeared similar to that of vessels perfused with native ferritin, which has been described in detail elsewhere (Loudon *et al.* 1979; Clough & Michel, 1981). The numbers and dimensions of the endothelial cell vesicles in cationized ferritin-perfused capillaries were similar to those reported by Clough & Michel in native ferritin-perfused vessels.

The most striking difference in the appearance of the capillaries perfused with cationized ferritin from those perfused with native ferritin was that a continuous layer of molecules of cationized ferritin was seen adhering closely to the luminal surface of the capillary endothelium. This layer partly filled some endothelial cell junctions and covered the necks of most of the endothelial cell vesicles (Pl. 1 *C*). In six capillaries perfused with cationized ferritin alone, prior to fixation with osmium tetroxide, the mean thickness of the layer was 26 ± 8·7 nm (mean ± S.E. of the mean). Addition of 1 % albumin to the cationized ferritin perfusate appeared to thicken the layer, which became more irregular. In five capillaries perfused with cationized ferritin and 1 % albumin prior to osmium fixation, the thickness of the layer ranged from 100 to 600 nm, having a mean thickness of 370 ± 214 nm (Pl. 1 *D*). Whilst it is possible that

serum albumin causes a thickening of the glycocalyx, possibly by ordering the glycoprotein matrix (Michel, 1981), it seems unlikely that it should do so to the extent observed. Large aggregates of cationized ferritin were seen in the capillary lumen after fixation, when albumin was present, and these were taken to indicate that the thickening of the cationized ferritin layer might be largely due to an artifact which caused cationized ferritin and albumin aggregates to adhere to the surface of the cell coat.

If a section thickness of 80 nm is assumed, estimates of the number of ferritin molecules in the layer attached to the cell surface were $6{\cdot}37 \pm 0{\cdot}22 \times 10^4$ molecules μm^{-3} (mean $\pm$ S.E. of the mean, $n = 24$) in the absence of albumin and $5{\cdot}98 \pm 0{\cdot}3 \times 10^4$ molecules μm^{-3} ($n = 20$) in its presence. Although the concentration of cationized ferritin in the layer appeared lower in the presence of albumin, it was not significantly so (Student's t). The number of molecules of cationized ferritin in a perfusate containing ferritin 4 g 100 ml^{-1} is approximately $3{\cdot}44 \times 10^4$ molecules μm^{-3}. It appears, therefore, that cationized ferritin not only binds to the cell surface of the perfused capillaries, but is also concentrated there by almost two-fold.

Plate 1 also shows electron micrographs taken of capillaries perfused with native ferritin. Native ferritin does not appear to bind to the cell surface, nor is it fixed in the capillary lumen in the absence of albumin (Pl. 1*A*). It has previously been demonstrated and is confirmed here that when albumin is present in the lumen, native ferritin is evenly fixed in the capillary lumen (Pl. 1*B*), and that the concentration of fixed native ferritin estimated from such electron micrographs is similar to that of the free ferritin in the perfusate (Loudon *et al.* 1979).

In these experiments native ferritin, although fixed in the lumen, appears to be excluded from a layer about 20 nm thick at the endothelial cell surface (Michel, 1981). This value approximates with that reported in the present study for the thickness of the layer of cationized ferritin adhering to the capillary wall. It suggests that in frog mesenteric capillaries, fixed after perfusion with solutions containing either native or cationized ferritin, the glycocalyx has an approximate thickness of 20–30 nm, and that it forms a continuous layer over the necks of most endothelial cell vesicles open to the capillary lumen and the endothelial cell junctions.

Labelling of endothelial cell vesicles

Endothelial cell vesicles at all three sites in the cell were seen labelled with molecules of cationized ferritin after perfusions lasting 60 s or longer. The fraction of each vesicle population labelled with cationized ferritin remained constant with lengthening times of perfusion, up to 240 s, confirming previous observations (Loudon *et al.* 1979; Clough & Michel, 1981, 1982). Table 1 shows the mean steady-state values of the fraction of each vesicle population labelled with one or more cationized ferritin molecules for capillaries perfused with either albumin-free (six experiments) or albumin-containing solutions (five experiments).

Similar data from capillaries perfused with solutions containing native ferritin in the presence and absence of albumin are also tabulated. These data are taken in part from Clough & Michel (1981). The four general features shown in the Table are as follows. (1) At all times there is a gradient of vesicles labelled with ferritin molecules across the cell; more luminal vesicles are labelled than cytoplasmic vesicles and more

cytoplasmic vesicles are labelled than abluminal vesicles, confirming that vesicles do participate in the transport of ferritin across the cell. (2) In the steady-state, all the luminal vesicles are labelled with molecules of cationized ferritin ($1{\cdot}0 \pm 0{\cdot}07$, $n = 899$), after corrections for sectioning losses. (3) Despite the heavy labelling of luminal vesicles, only 26% of the cytoplasmic vesicles are labelled with cationized ferritin in the steady-state. Abluminal labelling was also low, only 7% of the vesicles being labelled. The differences in the fractional labelling of vesicles across the cell are more marked in the present experiments than in those described by Clough & Michel (1981),

TABLE 1. Fractions of vesicle populations (N_L/N_T) labelled with one or more ferritin molecules, after perfusion for longer than 40 s with solutions of either native ferritin (6·7 g 100 ml^{-1}) or cationized ferritin (3–5 g 100 ml^{-1}) in the presence of albumin (1 g 100 ml^{-1}), and in its absence (L, luminal; C, cytoplasmic; A, abluminal)

	Native ferritin			Cationized ferritin		
	L	C	A	L	C	A
			(*a*) Ferritin alone			
N_L/N_T	0·84	0·41	0·25	1·00	0·26	0·07
±S.E.M.	0·07	0·04	0·05	0·07	0·04	0·03
N_T	309	352	125	899	876	374
			(*b*) Ferritin + 1% albumin			
N_L/N_T	0·60	0·39	0·11	0·33	0·09	0·04
±S.E.M.	0·04	0·02	0·02	0·05	0·02	0·005
N_T	303	437	210	427	425	257

where the fractions of cytoplasmic and abluminal vesicles labelled with native ferritin were much higher and that of the luminal vesicles significantly lower ($P < 0{\cdot}001$). Finally, (4) Table 1 shows that the addition of albumin to the cationized ferritin perfusate significantly reduces the fraction of vesicles labelled with ferritin at all sites in the cell, and that this effect is greater in cationized ferritin experiments than in those using native ferritin. The labelling of luminal, cytoplasmic and abluminal vesicles with cationized ferritin in the presence of albumin is reduced to a third of their values in its absence.

Concentration of cationized ferritin in luminal vesicles

Table 2 shows estimates of the mean values of F/N_L for luminal vesicles both in the presence and absence of albumin calculated using data from all the experiments, as described by Clough & Michel (1981), where

$$\text{mean } F/N_L = \frac{\Sigma \text{ (cationized ferritin molecules in all luminal vesicles)}}{\Sigma \text{ (labelled luminal vesicles)}}.$$

Also shown in Table 2 are the values of luminal F/N_L predicted, using the assumptions that (*a*) the luminal concentration of cationized ferritin is 4 g 100 ml^{-1}; and (*b*) the concentration of cationized ferritin available to the luminal vesicles is that estimated in the layer from electron micrographs (vide supra). If these values are compared with the value for luminal F/N_L observed, it is seen that the value estimated from the concentrations of cationized ferritin in the cell coat (5·78) is very

close to that observed (5·0). This suggests that cationized ferritin is not excluded from the luminal vesicles to the same extent as native ferritin and that, whilst the distribution of native ferritin appears limited by the glycocalyx lining the luminal vesicles (Clough & Michel, 1981), cationized ferritin is able to penetrate the glycocalyx and to distribute in a much larger fraction of the vesicle's volume. Luminal F/N_L falls to a value of $2{\cdot}04 \pm 0{\cdot}1$ ($n = 141$) in the presence of albumin, which is close to that seen for native ferritin, suggesting that under these conditions the exclusion of cationized ferritin from the luminal vesicles is increased.

TABLE 2. Cationized ferritin (c.f.) concentration in perfusate and in luminal vesicles

Luminal cationized ferritin (molecules μm^{-3})	No. of c.f. molecules expected in one vesicle	No. of c.f. molecules observed in one vesicle ± S.E.M.	
		no albumin present	1 % albumin present
Calculated from perfusate concentration (assuming a mol. wt. = 900000) $= 3{\cdot}44 \times 10^4$	3·0		
Calculated from electron micrographs (assuming section thickness of 80 nm) $= 6{\cdot}37 \times 10^4$	5·78	$5{\cdot}0 \pm 0{\cdot}7$ ($n = 899$)	$2{\cdot}04 \pm 0{\cdot}1$ ($n = 141$)

Transfer of ferritin to cytoplasmic and abluminal vesicles

Estimates of the steady-state distribution of cationized ferritin molecules within all the labelled vesicles from all the experiments (Fig. 1) show that, as expected from their higher F/N_L, luminal vesicles labelled with cationized ferritin have a more extensive range of labelling than cytoplasmic and abluminal vesicles and also than vesicles labelled with native ferritin. The distributions of cytoplasmic and abluminal labelling suggest that they arise as a result of dilution of the luminal distribution and that the cytoplasmic distribution is not derived from two separate distributions.

Values for the mean number of ferritin molecules per labelled cytoplasmic and abluminal vesicle, calculated as for mean luminal F/N_L, are shown in Table 3, together with the mean values of luminal F/N_L. These data confirm the previous finding of Clough & Michel (1981), using native ferritin, that cytoplasmic vesicles always contain fewer ferritin molecules than luminal vesicles. If these mean values of cytoplasmic F/N_L ($1{\cdot}87 \pm 0{\cdot}33$, $n = 228$) and luminal F/N_L ($5{\cdot}0 \pm 0{\cdot}7$, $n = 899$) are compared with those obtained using native ferritin (Table 3), it is seen that while cytoplasmic F/N_L falls to approximately 70 % of luminal F/N_L using native ferritin, with cationized ferritin cytoplasmic F/N_L is reduced to less than half of the mean value of luminal F/N_L.

Abluminal F/N_L with cationized ferritin ($1{\cdot}76 \pm 0{\cdot}39$, $n = 26$) is not sigificantly different from that of the cytoplasmic vesicles. Albumin reduces F/N_L at all three sites in the cell, but appears to have a lesser effect on cytoplasmic and abluminal F/N_L than it does on luminal F/N_L which falls to $2{\cdot}04 \pm 0{\cdot}1$ ($n = 141$).

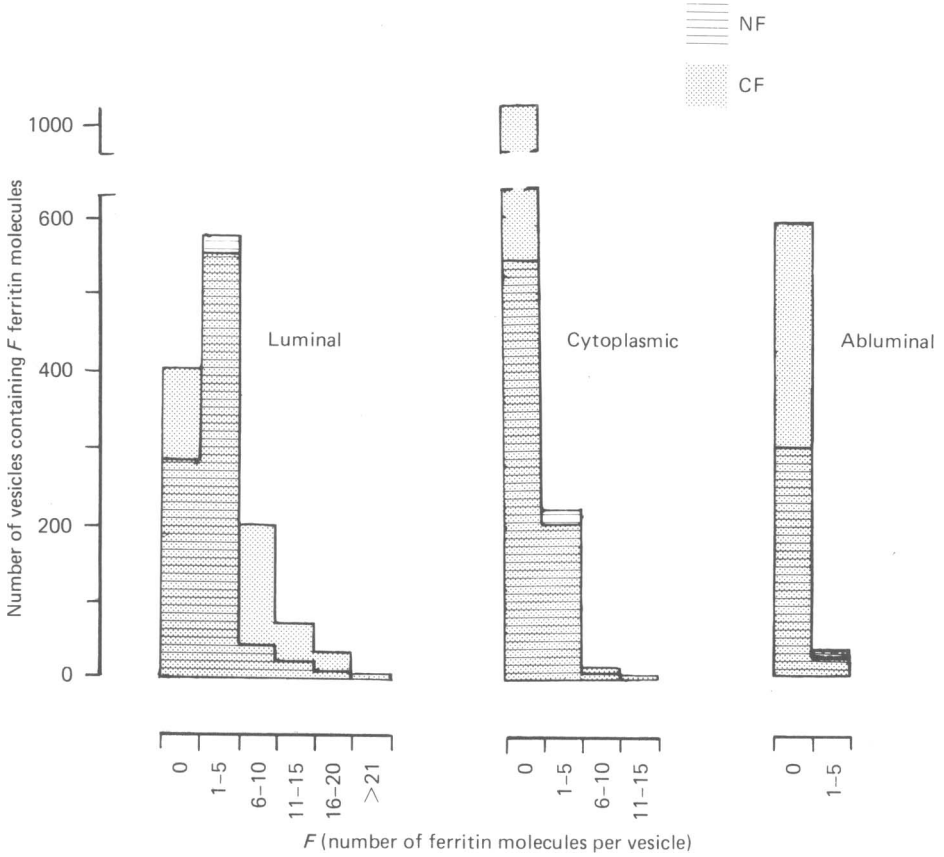

Fig. 1. Distribution of vesicles containing F ferritin molecules. Data taken from experiments in which capillaries were perfused with solutions of either native or cationized ferritin in Ringer or in Ringer containing bovine serum albumin 1 g 100 ml^{-1}, for periods exceeding 40 s. Number of vesicles counted in native ferritin experiments: luminal, 921; cytoplasmic, 789; abluminal, 336, and in cationized ferritin experiments: luminal, 1299; cytoplasmic, 1320; abluminal, 631.

TABLE 3. Ferritin contents of vesicles (molecules per labelled vesicle, F/N_L) after perfusion (> 40 s) with solutions of native (6·7 g 100 ml^{-1}) or cationized (3–5 g 100 ml^{-1}) ferritin, both in the presence of albumin (1 g 100 ml^{-1}) and its absence (L, luminal; C, cytoplasmic; A, abluminal)

	Native ferritin			Cationized ferritin		
	L	C	A	L	C	A
			(*a*) Ferritin alone			
Mean F/N_L	2·22	1·53	1·44	5·0	1·87	1·76
±S.E.M.	0·2	0·15	0·17	0·7	0·33	0·39
N_L	259	144	31	899	228	26
			(*b*) Ferritin + 1 % albumin			
Mean F/N_L	1·79	1·35	1·29	2·04	1·19	0·80
±S.E.M.	0·2	0·1	0·17	0·1	0·1	0·18
N_L	182	170	23	141	38	10

DISCUSSION

The steady-state distribution of molecules of cationized ferritin within the endothelial cell vesicles of single frog mesenteric capillaries perfused *in vivo* with solutions of cationized ferritin has again confirmed the involvement of vesicles in the transcellular transport of macromolecules (Jennings & Florey, 1967; Bruns & Palade, 1968; Casley-Smith & Chin, 1971; Loudon *et al.* 1979; Clough & Michel, 1981). The binding and concentration of cationized ferritin at the cell surface and the subsequent heavy labelling of endothelial cell vesicles has allowed a more detailed analysis of the mechanisms involved in this transfer of macromolecules by vesicles than has hitherto been possible. The patterns of labelling seen are wholly consistent with the model of macromolecular transport by the transient fusion of vesicles recently proposed by Clough & Michel (1981), and are not easily explained in terms of the more traditional view of transport by vesicle translocation (Palade, 1960; Mayerson *et al.* 1960; Renkin, 1964).

Initial labelling of luminal vesicles

Polycationic derivatives of ferritin bind to the surface of many types of cell both in glutaraldehyde pre-fixed preparations (Simionescu & Simionescu, 1978; Danon *et al.* 1972) and, occasionally, *in vivo* (Sprague *et al.* 1981; Simionescu & Simionescu, 1981). Whilst confirming this *in vivo* binding of cationized ferritin to the endothelium of frog capillaries, the present experiments have also shown that cationized ferritin can be concentrated there. The observation that cationized ferritin is taken up by the endothelial cell vesicles which are open to this layer is in direct contrast with that of Simionescu & Simionescu (1981), who reported cationized ferritin to be excluded by diaphragms covering the necks of vesicles of mouse jejunal and pancreatic endothelium, both *in vivo* and in pre-fixed tissue. It is supported by Sprague *et al.* (1981), who found cationized ferritin to enter the vesicles of the glutaraldehyde-fixed baboon arterial wall. These discrepancies are difficult to explain, particularly as diaphragms appear to be present, covering the necks of vesicles, in both frog mesenteric capillaries and in the arterial wall preparation.

The labelling of luminal vesicles with cationized ferritin in the steady-state is well described by a Poisson relationship, as is that with native ferritin (Clough & Michel, 1981), i.e. that the greater the average concentration of ferritin in the available vesicles, the greater the fraction of vesicles labelled. That 100 % of the luminal vesicles are labelled with cationized ferritin confirms Clough & Michel's finding that in the steady-state all luminal vesicles appear to have been through a cycle of availability at the cell surface. A comparison between the observed values of F/N_L within these labelled luminal vesicles and that predicted from the concentration of ferritin in the layer (Table 2) shows that these values closely correspond, and suggests that the glycocalyx within the vesicles is very similar to that covering the luminal cell surface and that cationized ferritin penetrates and binds to both. An interaction between albumin and this layer may account for the dramatic reduction in luminal labelling seen when serum albumin is added to the cationized ferritin perfusate. If albumin penetrates the layer and occupies space within it, or tightens the matrix of the layer by ordering the glycoprotein fibres (Clough & Michel, 1981; Michel, 1981) the volume

of distribution of cationized ferritin within the luminal vesicles will be reduced and N_L/N_T and F/N_L will fall. Alternatively, albumin might reduce luminal F/N_L and hence N_L/N_T by competing with cationized ferritin for specific binding sites within the matrix. On either hypothesis, the concentration of cationized ferritin in the surface layer might also be expected to decrease in the presence of albumin, if the cell coat lining the vesicles is similar to that covering the luminal cell surface. Although a small change in concentration was seen, it was not significant, and whilst larger changes might be masked by variations in thickness of the sections from which the concentration of ferritin was estimated, the possibility that the two cell coats are different cannot be ignored.

Wagner, Williams, Matthews & Andrews (1980) have also demonstrated that albumin inhibits the uptake of ferritin by vesicles in endothelium isolated from rat epididymal fat. Their finding that albumin does itself not enter the vesicles suggests that it is having its inhibitory effect on labelling at the cell surface, and not within the vesicles. Thus, in the present experiments, the presence of albumin in the cationized ferritin perfusate might be expected to reduce the number of vesicles labelled but not to affect the number of ferritin molecules within the vesicles which do label. As this is not the case, it is therefore possible that the two types of labelling seen involve two very different mechanisms, and should not be compared.

Transport of cationized ferritin across the cell

From studies on the labelling of endothelial cell vesicles with native ferritin, Clough & Michel (1981) proposed that transfer of ferritin by vesicles occured by the transient fusion and separation of neighbouring vesicles, and that during these fusions the contents of the vesicles mixed and equilibrated. One observation which led them to this proposal concerned the discrepancies between the steady-state values of N_L/N_T and F/N_L of cytoplasmic vesicles and those predicted by the translocation hypothesis. These discrepancies have been significantly increased in the present study using cationized ferritin and further support for Clough & Michel's proposal has thus been obtained.

The translocation hypothesis requires that cytoplasmic vesicles are derived half from the luminal cell surface and half from the abluminal cell surface, so that in the present experiments, if cytoplasmic vesicles are translocated luminal vesicles, cytoplasmic N_L/N_T should have a minimal value of 0·5 (half of the observed luminal N_L/N_T). That the observed cytoplasmic N_L/N_T (0·26) is so much lower than this implies that if vesicle translocation is occurring the fluxes of vesicles from the two surfaces are unequal, more than 80% of the cytoplasmic vesicles being derived from the abluminal population.

Secondly, according to the translocation model, it cytoplasmic vesicles were derived from luminal vesicles, in the steady-state their contents would be same as that of an average luminal vesicle. That the mean values of cytoplasmic F/N_L observed are always lower than those for luminal vesicles suggests that this is not so. The differences in F/N_L could only arise on the translocation model if the fluxes of vesicles were unequal (vide supra) and the majority (more than 80%) of cytoplasmic vesicles was derived from the albuminal vesicle population, whose F/N_L is not significantly different from that of the cytoplasmic vesicles. As such unequal

vesicle fluxes, of approximately 1:4, are not consistent with the translocation hypothesis (Tomlin, 1969; Shea, Karnovsky & Bossert, 1969; Green & Casley-Smith, 1972) alternative models have to be considered.

The present data are also inconsistent with the model proposed by Bundgaard *et al.* (1979). These authors have suggested that vesicles intercommunicate as stable structures, with 50% of cytoplasmic vesicles being continuous with the capillary lumen. If this model were correct, 50% of cytoplasmic vesicles should be labelled with cationized ferritin in the steady state and F/N_L should be the same in both luminal and cytoplasmic vesicles.

If, however, the model of Clough & Michel (1981) is adopted, the low level of labelling of cytoplasmic vesicles may be accounted for. Evidence has already been presented that cationized ferritin appears to bind to the cell coat lining the endothelial cell vesicles. Thus few 'free' ferritin molecules would therefore be available for exchange between vesicles and cytoplasmic N_L/N_T and F/N_L would be low despite high luminal N_L/N_T and F/N_L. The occasional appearance of a pair of fused vesicles, one full of molecules of cationized ferritin and the other not, offers morphological support for this argument. The increase in the labelling of cytoplasmic vesicles seen when albumin is present may similarly be explained if albumin increases the effective number of 'free' ferritin molecules available for exchange between luminal and cytoplasmic vesicles, by excluding ferritin from the layer.

This discussion leads to a similar picture of endothelial cell vesicles to that suggested by Clough & Michel (1981). In the steady-state all the vesicles open to the luminal cell surface have been through a cycle of availability to the luminal contents. When the lumen contains cationized ferritin all the luminal vesicles are labelled, cationized ferritin appearing not to be excluded from vesicles in the same way as native ferritin. Cationized ferritin is transported through the cell, not by the complete translocation of the vesicle and its contents, but rather by a series of fusions and seperations between neighbouring vesicles. However, cationized ferritin appears to bind to the cell coat lining the vesicles and is unable to distribute freely between the fused vesicles. Albumin increases the apparent exclusion of cationized ferritin from the vesicles, possibly by occupying space within the cell coat or by competing with cationized ferritin for specific binding sites within the glycoprotein matrix.

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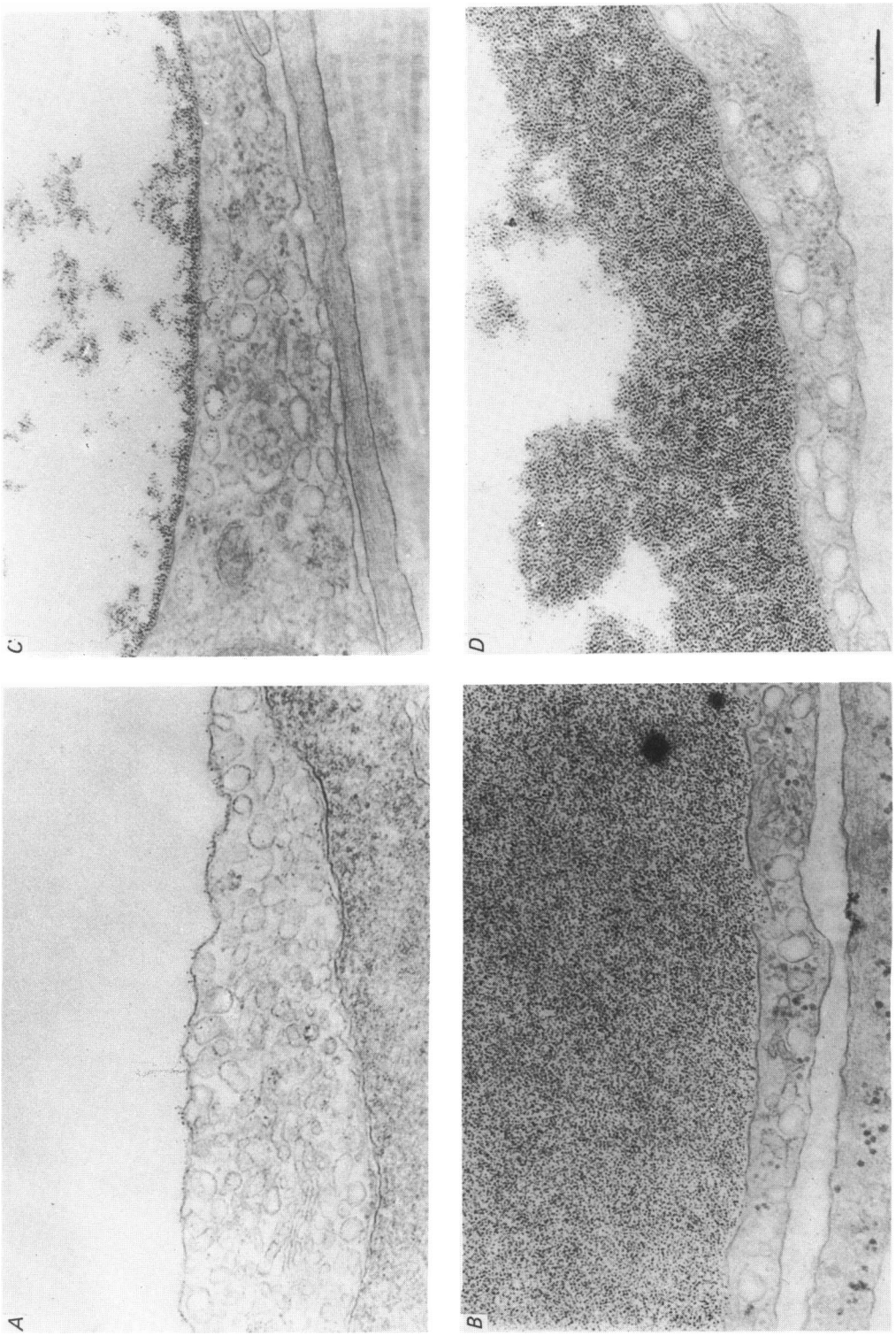

(*Facing p.* 401)

EXPLANATION OF PLATE

Electron micrographs of transverse sections of endothelium from capillaries perfused with *A*, native ferritin (6·7 g 100 ml^{-1}); *B*, native ferritin (6·7 g 100 ml^{-1}) and bovine serum albumin (1 g 100 ml^{-1}); *C*, cationized ferritin (3–5 g 100 ml^{-1}); and *D*, cationized ferritin (3–5 g 100 ml^{-1}) and bovine serum albumin (1 g 100 ml^{-1}). Capillaries were fixed *in situ* with 2 % osmium tetroxide after perfusion with ferritin for times exceeding 40 s. Calibration bar 0·2 μm.